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Method for investigating cytosine methylation in DNA sequences by means of triplex-forming oligomers

5   The present invention concerns a method for investigating cytosine methylation in DNA sequences. Triplex-forming oligomers are utilized, which preferably form triplex structures at positions where cytosine unmethylated at position 5 is present. The triplexes block the transcrip-

10   tion, replication and amplification of the DNA. In particular, peptide nucleic acid oligomers with modified nucleobases can be used as triplex-forming oligomers.

Background of the invention

15
5-Methylcytosine is the most frequent covalently modified base in the DNA of eukaryotic cells. It plays an important biological role, and is involved, among other things, in the regulation of transcription, in genetic

20   imprinting and in tumorigenesis (Millar et al.: Five not four: History and significance of the fifth base. In: The Epigenome, S. Beck and A. Olek, eds.: The Epigenome. Wiley-VCH Publishing Co. Weinheim 2003, pp. 3-20). The identification of 5-methylcytosine as a component of ge-

25   netic information is thus of considerable interest. It is difficult to detect methylation, of course, since cytosine and 5-methylcytosine have the same base pairing behavior. Many of the conventional detection methods based on hybridization are thus not capable of distinguishing

30   between cytosine and methylcytosine. In addition, the methylation information is completely lost in a PCR amplification.

The usual methods for methylation analysis operate essen-
35   tially according to two different principles. In the first case, methylation-specific restriction enzymes are

utilized, and in the second case, a selective chemical
conversion of unmethylated cytosines to uracil is con-
ducted (bisulfite treatment). The enzymatically or chemi-
cally pretreated DNA is then amplified for the most part
5   and can be analyzed in different ways (WO 02/072880 pp. 1
ff).

The method according to the present invention permits a
direct methylation analysis of the target DNA without
10   prior enzymatic or chemical pretreatment and is thus more
rapid and simpler than the conventional methodology. Ac-
cording to the invention, oligomers are utilized, which
bind sequence-specifically to the DNA and form triplex
structures therein.   The binding is preferably conducted
15   at positions at which unmethylated cytosine is present,
and this is predominantly caused by steric influences of
the methyl group of the 5-methylcytosine. The formation
of the triplex and hence the methylation status can be
detected in different ways. It is thus possible to detect
20   the unmethylated positions directly by use of labeled
triplex-forming oligomers. Methylation can be detected
via an amplification of the methylated DNA, while simul-
taneously, the amplification of unmethylated DNA will be
blocked by the triplex structure.
25

The invention utilizes the knowledge that DNA can be pre-
sent under certain circumstances in a triplex structure.
The triplex formation is promoted by a homopurine-
homopyridine sequence in the DNA double strand. A third
30   DNA strand can be deposited sequence-specifically in the
large groove of the double strand, whereby this gives
rise to the formation of hydrogen bonds with the ho-
mopurine sequence. Two different triplex structures oc-
cur, depending on the relative orientation of the third
35   strand of the purine strand. In the so-called pyrimidine
pattern, the third strand is rich in pyrimidine (Y) and

binds parallel to the purine (R) strand of the double he-
lix (Y-RY pattern). In this way, two Hoogsteen hydrogen
bonds are formed each time between thymine and adenine
(T-AT) as well as between protonated cytosine and guanine
(C$^+$-GC). The Y-RY triplexes are stable only under acidic
conditions. In the purine pattern one purine-rich third
strand binds anti-parallel to the purine strand of the
duplex (R-RY). Two reverse Hoogsteen base pairings result
between guanine and guanine (G-GC), adenine and adenine
(A-AT) or thymine and adenine (T-AT). The purine struc-
ture is independent of pH and more stable than the
pyrimidine pattern. The formation of both types of tri-
plexes is dependent on chain length, base composition,
concentration of divalent cations and temperature (Gun-
taka et al.: Triplex□forming oligonucleotides as modula-
tors of gene expression. Int J Biochem Cell Biol. 2003
Jan; 35 (1): 22-31).

Since it is necessary for the triplex formation that the
third strand interacts with a homopurine sequence in the
double strand, basically only G͟C and A͟T, and not C͟G und
T͟A base pairs can be detected in the double strand. The
triplex is destabilized by approximately 2.5-4.0 Kcal/mol
for each erroneous pairing; the stability of the triplex
formation is reduced by a factor of 10-100 or more
(Vasquez and Wilson: Triplex-directed modification of
genes and gene activity. Trends Biochem Sci. 1998 Jan; 23
(1): 4-9 1998 pp. 4, 5). Recently there have been various
attempts to extend the triplex recognition code to the
other two Watson-Crick base pairs. The highly promising
combinations are G-TA and T-CG. T-CG can occur both in a
parallel structure as well as in an antiparallel struc-
ture, whereas G-TA occurs only in the parallel form (Gow-
ers and Fox: Towards mixed sequence recognition by triple
helix formation. Nucleic Acids Res. 1999 Apr 1; 27 (7):
1569-77). Of course, these triplexes are also less sta-

ble than the canonical YRY triplexes. One reason for this
lies in the fact that in both cases only a single hydro-
gen bond is formed between T-CG and G-TA, whereas the ca-
nonical triads are stabilized by two hydrogen bonds. In
addition, the base stacking interactions appear to be de-
stabilized over long distances (Coman and Russu: Site-
resolved energetics in DNA triple helices containing G*TA
and T*CG triads. Biochemistry. 2002 Apr 2; 41 (13): 4407-
14, with further citations).

In the past, a pyrimidine recognition of the double
strand has been successfully achieved by chemical modifi-
cation of the bases of the triplex strand. For a cyto-
sine recognition within parallel triplexes, N^4-
substituted cytosine derivatives with side chains, which
also can form hydrogen bonds to guanine, are particularly
suitable (Gowers and Fox 1999, loc. cit., p. 1573 with
further citations; Vasquez and Glaser: Triplex-forming
oligonucleotides: principles and applications. Q. Rev.
Biophys. 2002 Feb; 35 (1): 89 ff, 98). Particularly suit-
able are N^4-(3-acetamidopropyl)cytosine and N^4-(6 amino-
2-pyridinyl)cytosine (Figs. 1 and 2).

In addition to modified bases, a plurality of modifica-
tions of the third strand have been developed in order to
stabilize triplexes and reduce their degradation in cells
and tissues. Thus, in the case of the triplex-forming
oligonucleotides which were used, the phosphorus atoms in
the phosphate backbone have been replaced by sulfur, the
OH groups in the ribose and the purine rings have been
methylated or the 5' and 3' ends have been blocked by
different components (Guntaka et al. 2003, loc. cit., p.
23 with further citations). Other triplex-forming mole-
cules are also used, particularly peptide nucleic acids
(PNA). It is interesting in this regard that very stable
triplexes can be formed from triplex-forming PNAs with

DNA-PNA duplexes (PNA$_2$-DNA triplexes; Ray and Norden:
Peptide nucleic acid (PNA): its medical and biotechnical
applications and promise for the future. FASEB J. 2000
Jun; 14 (9): 1041 ff, 1048).

The triplex formation is the foundation of several medi-
cal and biological applications, e.g., for transcription
modulation, for site-directed mutagenesis, for promoting
recombination, and for inhibiting polymerases (Guntaka et
al. 2003, loc. cit. p . 26 f, Vasquez and Glaser 2002,
loc. cit. p . 98 ff, each with further citations).

Description of the invention

The object of the present invention is to provide a novel
method for the detection of methylated positions.

The object is solved by the characterizing features of
the main claim. Advantageous enhancements of the method
according to the invention are characterized in the de-
pendent subclaims.

The object is solved by a method for the detection of cy-
tosine methylations in DNA, wherein the DNA to be inves-
tigated is brought into contact with a triplex-forming
molecule, which distinguishes between methylated and un-
methylated DNA.

According to the invention, it is preferred that the tri-
plex-forming molecule forms a triplex with the DNA to be
investigated, whereby the triplex formation is preferred
for unmethylated DNA as opposed to triplex formation for
methylated DNA, and the triplex formation is used for the
detection of the methylation status.

According to the invention, it is also preferred that
oligonucleotides, peptide nucleic acid (PNA) oligomers,
other oligonucleotide analogs or chimeras, or molecules
derived from these substance classes are used as the tri-
5   plex-forming molecules.

It is further preferred that the triplex-forming molecule
bears both a duplex-forming as well as also a triplex-
forming sequence.

10

It is also preferred that the triplex-forming molecule
bears at least one modified nucleobase, which specifi-
cally or selectively binds to a cytosine in the triplex.
It is further preferred that N^4-substituted cytosine de-
15   rivatives are used as the nucleobase. It is also pre-
ferred here that N^4-(3-acetamidopropyl)cytosine or N^4-(6-
amino-2-pyridinyl)cytosine is used as the nucleobase. It
is most particularly preferred that N^4-substituted cyto-
sines which bear additional modifications at position 3
20   are used as the nucleobase. It is particularly preferred
also that position 3 is modified with a methyl, ethyl or
isopropyl group.

A method in which the triplex-forming molecule bears a
25   detectable label is also preferred according to the in-
vention.

A method is preferred, in which the methylation status is
detected via an in-situ hybridization.

30

In addition, a method is preferred, in which the DNA is
amplified for detection of the methylation status,
wherein the methylated DNA is preferentially amplified
over unmethylated DNA due to the triplex formation.

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A method is also particularly preferred, in which the DNA
is amplified for detection of the methylation status,
wherein unmethylated DNA is preferentially amplified over
methylated DNA due to the triplex formation.

It is particularly preferred that triplex-forming mole-
cules are utilized, which also serve as primers in the
amplification.

A method is also preferred, in which structures which
hinder an amplification are formed by the triplex forma-
tion.

It is particularly preferred that deoxy-5-methylcytosine
triphosphate, and not deoxycytosine triphosphate (dCTP),
is utilized in the amplification.

In addition, it is preferred that a real-time PCR is
utilized for the amplification.

According to the invention, a method is also provided for
separating methylated and unmethylated DNA, wherein
(a) the DNA is brought into contact with a triplex-
forming molecule,
(b) the triplex-forming molecule forms a triplex with the
DNA, wherein triplex formation is preferred with unmethy-
lated DNA over triplex formation with methylated DNA, and
(c) the triplex formation is utilized for the separation.

According to the invention, a method is also provided for
the specific introduction of DNA damage into unmethylated
DNA, wherein
(a) the DNA is brought into contact with a triplex-
forming molecule, which bears a reactive chemical group,

(b) the triplex-forming molecule forms a triplex with the
DNA, wherein triplex formation with unmethylated DNA is
preferred over triplex formation with methylated DNA,

(c) one reacts the reactive chemical group with the DNA
present in the triplex form.

In addition, according to the invention, a method is also
provided for the specific inhibition of replication of
unmethylated DNA, wherein

(a) the DNA is brought into contact with a triplex-
forming molecule,

(b) the triplex-forming molecule forms a triplex with the
DNA, wherein triplex formation with unmethylated DNA is
preferred over triplex formation with methylated DNA,

(c) the replication of the DNA present in the triplex
form is inhibited.

According to the invention, a method is also provided for
the specific inhibition of the transcription of unmethy-
lated DNA, wherein

(a) the DNA is brought into contact with a triplex-
forming molecule,

(b) the triplex-forming molecule forms a triplex with the
DNA, wherein triplex formation with unmethylated DNA is
preferred over triplex formation with methylated DNA,

(c) the transcription of the DNA present in the triplex
form is inhibited.

Another subject of the present invention is the use of
oligonucleotides, peptide nucleic acid (PNA) oligomers,
other oligonucleotide analogs or chimeras, or molecules
derived from these classes of substances, which contain
N^4-(3-acetamidopropyl)cytosine, N^4-(6-amino-2-
pyridinyl)cytosine or other N^4-substituted cytosine de-
rivatives, for the therapy of disorders which are associ-
ated with demethylation of cytosines.

The method according to the invention for distinguishing between methylcytosine and cytosine utilizes steric hindrance, which proceeds from the methyl group of the methylcytosine and which can prevent the binding of specific triplex-forming oligomers. In addition to steric considerations, electronic influences of the methyl group probably also play a role. Both oligonucleotides and peptide nucleic acid (PNA) oligomers can be utilized as triplex-forming oligomers. The use of other oligonucleotide analogs or chimeric molecules derived from the above-named classes of substances is also possible. The binding code for the third strand and the preferred conditions under which a triplex formation occurs are prior art (see above; see also the citations in US 6,461,810 B1, column 3, lines 30ff). For cytosine detection, it is considered that basically an interaction of the third strand with a homopurine sequence is necessary for the triplex formation, but cytosine is a pyrimidine base. Therefore, the use of a modified base is necessary for cytosine detection. N^4-substituted cytosine derivatives with side chains that also form hydrogen bonds to guanine are particularly suitable within parallel triplexes and thus increase the stability of the triplex. For distinguishing between cytosine and methylcytosine, it is necessary that the modified bases are structured in such a way that a triplex formation is made difficult sterically by the 5-methyl group of the cytosine. N^4-(3-acetamidopropyl)-cytosine and N^4-(6-amino-2-pyridinyl)cytosine, e.g., are particularly preferred as modified bases. The production of these bases is prior art (Gowers and Fox 1999, loc. cit., p. 1573 with further citations). It is to be expected that the steric hindrance and thus the capacity for distinguishing between cytosine and methylcytosine is further increased, if the utilized N^4-substituted cyto-

sine derivatives bear additional modifications at posi-
tion 3, e.g., methyl, ethyl or isopropyl substituents.

A plurality of other modifications of the sugars and
5   backbone of the nucleotides, which can be utilized in or-
der to stabilize the triplex, also belong to the prior
art. In particular, peptide nucleic acid (PNA) oligomers
are used (Guntaka et al. 2003, loc. cit., with further
citations). It is also known that the triplex formation
10   can be stabilized by intercalators or triplex-specific
ligands (Sun: New targets for triple helix forming oli-
gonucleotides. pp. 273 ff, 276; Escude and Garestier:
Triple helix stabilizing agents pp.257 ff, each found in:
C. Malvy, A. Harel-Bellan, LL Pritchard, eds: Triple he-
15   lix-forming oligonucleotides. Kluwer Academic Publishers
1999).

The DNA to be investigated can be present in single as
well as double strands. In double-stranded DNA , oligonu-
20   cleotides are preferably utilized as triplex-forming
molecules. If one starts with single-stranded DNA, then
first, a duplex formation must precede the triplex forma-
tion. Both steps can be mediated by the same molecule,
if this molecule bears a sequence that is complementary
25   to the target DNA and also provides a triplex-forming do-
main. It is particularly preferred here to utilize PNA
molecules ("bis-PNA"), since PNA_2-DNA-triplexes are par-
ticularly stable (Ray and Norden 2000, loc. cit. p.
1048). The orientation of the Watson-Crick PNA strand is
30   thus antiparallel to the DNA, while the orientation of
the Hoogsteen strand is parallel to the DNA. Both se-
quences are coupled together via a flexible linker. In-
formation on the type and length of the linker are de-
scribed, e.g., in US 5,693,773 (column 7, lines 64 ff).
35   For the formation of parallel triplexes, a protonation of
the cytosines in the third strand is necessary. This pH

dependence can be circumvented, however, if the cytosines
are replaced by pseudoisocytosines (J). The triplex-
forming PNA molecules then contain cytosine in the Wat-
son-Crick sequence and pseudoisocytosine in the Hoogsteen
5   sequence. Bis-PNA molecules can also be utilized for the
investigation of double-stranded DNA. Thus the duplex-
forming strand of the PNA displaces the corresponding DNA
double strand (see: Bentin and Nielsen: Triplexes involv-
ing PNA, in: C. Malvy, A. Harel□Bellan, LL Pritchard,
10   eds: Triple helix-forming oligonucleotides. Kluwer Aca-
demic Publishers 1999, pp. 245 ff with further cita-
tions).

The methylation-dependent triplex formation can be de-
15   tected in different ways. According to the invention, the
triplex-forming molecule is to be labeled and then the
triplex, thus the unmethylated state, is to be detected.
A preferred embodiment of this method is in-situ hybridi-
zation. Unmethylated double-stranded DNA can be detected,
20   e.g., in cells in this way. The sequence specificity of
the method according to the invention is thus a particu-
lar advantage when compared with other known methods for
the in-situ detection of cytosine methylations, e.g., via
methylation-specific antibodies.

25

In particular, chemically modified oligonucleotides can
serve as triplex-forming molecules (see above). The oli-
gonucleotides are between 7 and 50 nucleotides, prefera-
bly between 10 and 30 nucleotides long. They bear re-
30   porter molecules, which can be detected with chemical or
physical methods, e.g., biotin, fluorescence or radioac-
tive labels. The exact conditions for an in-situ hybridi-
zation with triplex formation are prior art (US 6,461,810
B1, particularly columns 3 ff).

35

In another variant according to the invention, the detection of methylation is made via an amplification of the methylated DNA, while simultaneously the amplification of unmethylated DNA is blocked by the triplex structure.

5 Current methods, e.g., PCR, can be employed for the amplification. The binding of primers is independent of the methylation status, but the extension of the primers is blocked by the triplex formation at specific sites. It is known that DNA polymerases are not able to decompose tri-

10 plex structures. The polymerization thus comes to a halt at these sites (WO 96/18732, particularly page 18, lines 17 ff). Due to the particular stability of the triplex, it is preferred to use the above-mentioned bis-PNA molecules, which bear both duplex-forming as well as the tri-

15 plex-forming sequences, for such blocking. Of course, two different molecules or oligonucleotides can also be used. It is also conceivable and preferred to use other oligonucleotide analogs, e.g., LNA (Locked Nucleic Acids). Methods are known to the person skilled in the art for

20 the production of corresponding oligonucleotide analogs (Braasch and Corey: Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. Chem Biol 2001 Jan;8(1):1-7 with further citations).

25 The concentration of the blocker molecules must be high enough so that a complete blocking is ensured. A concentration range between 100-1000 nM is preferred. The amplification of the methylated DNA is otherwise performed according to the prior art. Care is to be taken, of

30 course, that only 5-methylcytosine nucleoside triphosphates should be utilized. If cytosine were incorporated in the newly synthesized DNA strands, then the triplex-forming molecules would not only bind to the originally unmethylated DNA, but also to the new molecules produced

35 from the originally methylated DNA. The amplificates can be detected in different ways known to the person skilled

in the art, e.g., by methods of length measurement such
as gel electrophoresis, capillary electrophoresis and
chromatography (e.g., HPLC). Also real-time variants can
be utilized, e.g., the Taqman or the Lightcycler method.

Another embodiment according to the invention for the se-
lective amplification of methylated DNA lies in the use
of primers which simultaneously provide a triplex-forming
domain. Oligonucleotides are preferably used for this
purpose. The Watson-Crick-forming sequence here lies at
the 3'-end of the primer, and the Hoogsteen-forming se-
quence lies at the 5'-end. As described above, both parts
are joined via a linker. The primers are between 30 and
80 nucleotides long, and they are preferably more than
40-60 nucleotides. Amplification can only occur if the
primer does not form a triplex, thus if the corresponding
cytosine position is methylated. Again, care must be
taken to use methylated deoxycytosine triphosphate in the
amplification. The amplificates can be detected as de-
scribed above. According to the invention, the opposite
variant is also provided, in which the primers are con-
structed in such a way that an extension will only be
produced if a triplex is formed when the cytosine posi-
tion is in the unmethylated form.

In addition to the detection of methylated DNA, the
method according to the invention can also be used in or-
der to separate methylated sequences from unmethylated
ones. Thus, if (a) the DNA is brought into contact with a
triplex-forming molecule,
(b) the triplex-forming molecule forms a triplex with the
DNA, wherein triplex formation with unmethylated DNA is
preferred over triplex formation with methylated DNA, and
(c) the triplex formation is utilized for the separation.

A preferred possibility for this procedure is the triple
helix affinity chromatography described in the literature
(see: Kamenetskii: Triplexes and biotechnology. In: C.
Malvy, A. Harel-Bellan, LL Pritchard, eds: Triple helix-
forming oligonucleotides. Kluwer Academic Publishers
1999, 285, 287 ff with further citations).

The methylation-specific triplex formation can also be
utilized for further applications. One possibility ac-
cording to the invention is the sequence-specific intro-
duction of DNA damage into unmethylated DNA. Here,
(a) the DNA is brought into contact with a triplex-
forming molecule, which bears a reactive chemical group,
(b) the triplex-forming molecule forms a triplex with the
DNA, wherein triplex formation with unmethylated DNA is
preferred over triplex formation with methylated DNA,
(c) the reactive chemical group reacts with the DNA pre-
sent in triplex form.

Psoralens or alkylating reagents are particularly pre-
ferred as reactive chemical groups. The precise condi-
tions of comparable methods are described in the litera-
ture (Faria and Giovannangeli, Triplex-forming molecules:
from concepts to applications. J Gene Med. 2001 Jul-
Aug;3(4):299-310 with further citations).

Other applications of the invention are transcription
modulation, replication inhibition, site-directed
mutagenesis and promoting the recombination of specific
unmethylated DNA sequences. Here,
(a) DNA is brought into contact with a triplex-forming
molecule,
(b) the triplex-forming molecule forms a triplex with the
DNA, wherein triplex formation with unmethylated DNA is
preferred over triplex formation with methylated DNA, and

(c) the replication or the transcription of the DNA present in triplex form is inhibited, or mutations or recombinations are promoted.

5   The precise conditions of comparable methods are described in the literature (Faria and Giovannangeli, Triplex-forming molecules: from concepts to applications. J Gene Med. 2001 Jul-Aug;3(4):299-310 with further citations).

10

According to the invention, the above-named methods can also be used therapeutically. It is known that many disorders are associated with cytosine demethylation of promotor regions or other regulatory regions of specific

15   genes. There is an activation of transcription due to this demethylation. The application of triplex-forming molecules permits a sequence-specific inhibition of transcription or replication or a targeted damage of the corresponding sequences. In particular, oligonucleotides,

20   peptide nucleic acid (PNA) oligomers, other oligonucleotide analogs or chimeras, or molecules derived from these classes of substances, are considered as possible therapeutic agents, which contain N^4-substituted cytosine derivatives, e.g., N^4-(3-acetamidopropyl)cytosine or N^4-(6-

25   amino-2-pyridinyl)cytosine. The oligomers can be administered together with a pharmaceutical vehicle and possibly via different pathways with additional adjuvants. It is also possible to combine them with other therapeutic agents. The precise composition and the type of admini-

30   stration can be determined by the person skilled in the art according to conventional pharmaceutical principles.

Brief description of the Figures:

Figure 1 shows N^4-(3-acetamidopropyl)cytosine and the
formation of hydrogen bonds with a C-G pair. The figure
is taken from Gowers and Fox 1999, loc. cit.

Figure 2 shows N^4-(6-amino-2-pyridinyl)cytosine and the
formation of hydrogen bonds with a C-G-pair. The figure
is taken from Gowers and Fox 1999, loc. cit.

                            EXAMPLES

Example 1:
Detection of cytosine methylations by use of bis-PNA
molecules.

The underscored cytosines presented in the following DNA
sequence will be detected in their methylated state, but
not in their unmethylated state. Detection is made by
PCR, in which the presented sequence is amplified in the
presence of this methylation, but not in its absence.

5'-
CGGAGGAAGAAAGAGGAGGGGCTGGCTGGTCACCAGAGGGTGGGGCG-
GACCGCGTGCGCTCGGCGGCTGCGGAGAGGGGGAGAGCAGG-
CAGCGGGCGGCGGGGAGCAGCATGGAGCCGGCGGCGGGGAGCAGCATGGAGCCTT-
3' (SEQ ID: 1)
For this purpose, isolated and purified DNA is subjected
to PCR, with the use of the primers CGGAGGAAGA-AAGAGGAG
and AAGGCTCCATGCTGCTC (both at concentrations of 300 nM
each), in the presence of 100 nM of the bis-PNA molecule
NH2-CTCCCCGCCGC-O-O-O-JXJJXJJJJTJ-COOH (X = N^4-(3-
acetamidopropyl)cytosine; 0 = 8-amino-3,6-dioxaoctanoic
acid; J = pseudoisocytosine) in a reaction volume of 0.02
ml (55°C annealing temperature, 72°C primer extension
temperature, 95°C denaturing temperature, 42 reaction cy-

cles). Deoxy-5-methylcytosine triphosphte is utilized in-
stead of deoxycytosine triphosphate. The amplificate
with a length of 143 base pairs is detected after separa-
tion of the PCR product by agarose gel electrophoresis in
5   the presence of ethidium bromide by visualization in ul-
traviolet light.

The modified cytosine is synthesized as described in the
literature (Gowers and Fox 1999, loc. cit., p. 1573 with
10   further citations). The synthesis of the PNA molecules is
also prior art (see also the citations in Ray and Norden
2000, loc. cit., p. 1042).

Example 2:
15   Detection of cytosine methylations with the use of PNA-
DNA hybrid molecules

The underscored cytosines presented in the following DNA
sequence will be detected in their methylated state, but
20   not in their unmethylated state. Detection is made by
PCR, in which the presented sequence is amplified in the
presence of methylation, but not in its absence. For this
purpose, a PCR primer with a PNA domain is modified in
such a way that when methylation is absent, but not when
25   it is present, a triplex structure is formed, which pre-
vents the binding of the DNA polymerase and thus the am-
plification.

5′-CGGAGGAAGAAAGAGGAGGGGCTGGCTGGTCACCAGAGGGTGGGG-
30   CGGACCGCGTGCGCTCGGCGGCTGCGGAGAGGGGGAGAGCAGG-
CAGCGGGCGGCGGGGAGCAGCATGGAGCCGGCGGCGGGGAGCA-3′
(SEQ. ID: 2)

Isolated and purified DNA is subjected to PCR with the
35   use of the primer CGGAGGAAGAAA-GAGGAG and the PNA-DNA hy-
brid molecule (both 300 nM) NH2-XJJXJJXJJJJTJ-O-O-O-O-

(T)GCTCCCCGCCGCCG-3' (DNA monomers in italics; X = N^4-(3-
acetamidopropyl)cytosine; O = 8-amino-3,6-dioxaoctanoic
acid; J = pseudoisocytosine; (T) = 5'-aminothymidine) in
a reaction volume of 0.02 ml (55°C annealing temperature,
5   72°C primer extension temperature, 95°C denaturing tem-
perature, 42 reaction cycles). Deoxy-5-methylcytosine
triphosphate is utilized as the cytosine deoxynucleoside
triphosphate. The amplificate with a length of 143 base
pairs is detected after separation of the PCR product by
.10   agarose gel electrophoresis in the presence of ethidium
bromide by visualization in ultraviolet light.

The synthesis of PNA-DNA hybrid molecules is prior art
(see, e.g., Uhlmann et al. Angew. Chem. 1998, 110, 2954-
15   83 and the citations contained therein).

Example 3:
Methylation-specific labeling of DNA by use of bioti-
nylated bis-PNA molecules
20

The following sequence will be labeled with a biotin
group in the region of the two underscored cytosines via
the binding of a bis-PNA only if these cytosines are pre-
sent in their unmethylated state, but not if they are in
25   their methylated form.

5´-
CGGAGGAAGAAAGAGGAGGGGCTGGCTGGTCACCAGAGGGTGGGGCG-
GACCGCGTGCGCTCGGCGGCTGCGGAGAGGGGGAGAGCAGG-
30   CAGCGGGCGGCGGGGAGCAGCATGGAGCCGGCGGCGGGGAGCAGCATGGAGCCTT-
3´ (SEQ ID 3)
For this purpose, isolated and purified DNA is dissolved
in 100 mM phosphate buffer (pH 7-8, 500 mM NaCl) contain-
ing 100 nM biotinyl-NH-CTCCCCGCCGC-O-O-O-JXJJXJJJJTJ-COOH
35   (X = N^4-(3-acetamidopropyl)cytosine; O = 8-amino-3,6-
dioxaoctanoic acid; J = pseudoisocytosine) and, after a

ten-minute denaturing at 95°C, is incubated for three
hours at 55°C. After separation of the excess bis-PNA,
for example, by ultrafiltration or gel filtration, the
labeled DNA is available for further investigations.

5